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ORIGINAL PAPER

Characterization of *Ostertagia ostertagi* annexin-like proteins at different developmental stages

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Abstract Ostertagiosis remains an economically important parasitic disease in cattle in the temperate regions of the world. Repeated exposures to *Ostertagia ostertagi* in calves cause significant pathology in the abomasum but elicit little protective immunity. The larvae use the host's gastric glands as a niche for development, where the parasite completes its parasitic stages, while in the gastric glands, the larvae must down-regulate the host inflammatory immune responses. Annexin (ANX) A1, commonly found in most eukaryotes, is heavily involved in controlling anti-inflammatory responses by binding receptors on leukocytes. We hypothesized, therefore, that parasite proteins of the ANX family may be involved in host-parasite interactions during ostertagiosis. BLASTN search with the bovine ANXA1 identified two families of Oos-ANX like proteins (Oos-ANXL), each of which was highly conserved at the genetic level and identical at the amino acid sequence level. Oos-ANXL-1 is encoded by two transcripts and Oos-ANXL-2 by 20 transcripts. The present study characterized one Oos-ANXL, representing the most abundant Oos-ANXL, which was further defined as Oost-ANXL-2.1. Oos-ANXL-2.1 with a coding sequence of 519 bp was PCR-amplified, cloned, and expressed. Oos-ANXL-2.1 was immunolocalized to both L3 and adult, but not L4. The staining appeared to be associated with the gut and hypodermis in L3, but it was specifically localized to the hypodermis in adult worms. Western blots detected three protein bands in parasite lysates using anti-recombinant Oos-ANXL-2.1 antibody. Integrated optical density for each of the 3 Oos-ANXL-2s or the total Oos-ANXL-2s detected by Western blots ($P < 0.05$) was higher in adult worms than in L3 or L4. The results indicate that the production of Oos-ANXL-2s is developmentally regulated and most abundant in the adult worm. This rather large family of proteins could be a potential vaccine target against *O. ostertagi* infection and warrants further investigation.

Keywords *Ostertagia ostertagi* · Ostertagiosis · Cattle · Gastric glands · Annexin · Immune modulator

Introduction

Bovine ostertagiosis is caused by *Ostertagia ostertagi*, a nematode parasite, and considered a major economic burden to the livestock industry. The infection occurs normally in the first and second year grazing seasons of young animals. Ostertagiosis can cause severe pathologies in the abomasum, leading to reduced weight gain or weight loss and decreased feed conversion (Fox 1993; Gibbs 1988). It is known that the causative parasite, *O. ostertagi*, survives within the cattle host by down-regulating host immune responses by releasing excretory-secretory products (ES) that mimic the host immune modulators and recognize the complementary cellular receptor but exert functions that benefit parasite's survival.

Annexins (ANXs) are a diverse group of proteins found in plants, animals, and fungi and capable of binding to negatively charged phospholipids. These proteins have been shown to involve in a number of calcium-regulated functions associated with the membrane. ANXs play a critical role in various cellular and physiological processes by providing a membrane scaffold to retain cell shape, and it may also function as membrane-membrane or membrane-cytoskeleton linkers

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(Rescher and Gerke 2004; Creutz 1992). Extracellular ANXs are associated with conditions including fibrinolysis, coagulation, inflammation, and apoptosis (van Genderen et al. 2008; Perretti and Gavins 2003; Parente and Solito 2004). ANXs contain a 60–70 amino acid repeat sequence (ANX repeats), which contains calcium and phospholipid binding sites (Clark et al. 2001). Four ANX-like proteins in *Caenorhabditis elegans* with difference in their expression profile and ligand-binding properties were identified (Nishioka et al. 2007). Multiple studies have reported ANXs with immunomodulatory effects. ANXs have been identified as an immunodominant component of the membrane lipopolysaccharide core region of intestinal parasites such as *Giardia lamblia* (Weiland et al. 2003), *Schistosoma mansoni* (Braschi et al. 2006), and *Taenia solium* metacestodes (Zhang et al. 2007). Further, secretion of ANX-B1 from *T. solium* was shown to induce apoptosis in eosinophils (Yan et al. 2008). Pigs vaccinated with ANX-B1 exhibited a decrease in the number of cysticerci by 85% (Guo et al. 2004). In the present study, we have identified multiple Oos-*anxl* transcripts in *O. ostertagi*, which closely resemble the *nex*-2 and -3 gene sequences of *Caenorhabditis remanei*. In the present study, we cloned and expressed the Oos-*anxl*-2.1 gene in *Escherichia coli* and purified the protein to homogeneity by affinity chromatography. The Oos-ANXL-2s was characterized for spatial localization and relative expression in the parasite at different developmental stages using an antibody raised against the purified recombinant Oos-ANXL-2.1.

Materials and methods

Identification of gene(s) encoding Oos-*anx-like* (Oos-*anxl*) proteins

The *O. ostertagi anxl* genes were identified by BLASTN search with bovine *anxa1* (http://www.nematode.net). The transcripts for the genes most similar to *anxa-1* were retrieved and compared by multiple sequence alignment using the Molecular Evolutionary Genetics Analysis (MEGA) tool. The open reading frames (ORF's) of *anxl* genes were predicted using ORF Finder tool available at NCBI (http://www.ncbi.nlm.nih.gov/gorf/gorf.html). The predicted ORFs were translated into protein sequences using the ExPASy translate tool (http://web.expasy.org/translate/). A nucleotide sequence of Oos-*anxl* representing the most abundant Oos-*anxl* was used for cloning and protein expression. The signal peptide in Oos-ANXL proteins was predicted using ProP 1.0 server (http://www.cbs.dtu.dk/services/ProP/). The conserved ANX domains in the putative ANXL proteins were predicted using protein sequence analysis and classification tool (InterPro 58.0, https://www.ebi.ac.uk/interpro/).

Cloning of the Oos-*anxl* genes

Total RNA was extracted from adult *O. ostertagia* using the RNeasy Mini Kit (Qiagen, Hilden, Germany), and cDNA was synthesized using oligo dT (20mer) primers (IDT, Inc., Coralville, Iowa) and the M-MuLV Reverse Transcriptase Kit (New England Biolabs, Ipswich, MA) according to the manufacturer's instructions. Oos-*anxl* gene was PCR-amplified from the cDNA using Oost-*anxl* primer pairs (forward primer: 5′-caaatgggtcgcGGATCCATGTTGGTGCTTCTTCTGCAAGGC-3′, reverse primer: 5′-*agctcgaattc*GGATCCTCAATAAAGACTTCTGTTGCCTTTGACGATG-3′), which were designed based on the 5′ and 3′ conserved regions of all predicted ORFs. The primers were designed for cloning by the In-Fusion® HD cloning kit (Takara Bio USA, Inc., Mountain view, CA). In the primer sequences, underlined sequence is the Bam H1 cleavage site which is used to linearize the vector, and the primer sequences in lower case are complimentary to the vector sequences flanking the Bam H1. Primer sequences following the Bam H1 site are Oos-*anxl-2*-specific. The conditions used for PCR reaction were as follows: 98 °C for 1 min followed by 35 cycles at 98 °C for 20 s, 55 °C for 20 s, 72 °C for 60 s with a final extension of 72 °C for 5 min. The PCR-amplified product was purified using PCR purification kit (Promega, Madison, WI) and cloned into pET28a (+) expression vector (EMD Millipore, Billerica, MA), and the construct was transformed into *Escherichia coli* DH5α. The recombinant plasmid (pET28a-Oos-*anxl*) was extracted from *E. coli* DH5α using Wizard® Plus SV Mini preps (Promega, Madison, WI), and the construct was verified by sequencing prior to protein expression studies.

Expression, purification, and antibody production

The recombinant construct, pET28a-Oos-*anxl*, was transformed into *E. coli* BL21 (DE3) and grown overnight at 37 °C in Luria-Bertani (LB) broth containing kanamycin (50 μg/ml). The next day, fresh LB medium (500 ml) containing the antibiotic was inoculated with the overnight culture (5 ml), grown to OD_{600} = 0.6, and induced with isopropyl β-D-1-thiogalactopyranoside (IPTG) (100 μM) for 14 h. The next day, the cells expressing rOos-ANXL-2.1 were centrifuged, solubilized in a 8 M guanidine-HCl, and purification by Ni-NTA affinity chromatography (Qiagen, Hilden, Germany). The molecular mass and purity of the recombinant protein were determined by 4–12% SDS-PAGE (NuPAGE, Thermo Fisher, Halethorpe, MD) and used for antibody production in rabbits (Pacific Immunology, Ramona, CA).

Western blotting

Western blot analysis was performed as described previously (Qu et al. 2013). Briefly, samples were separated on a 4–12%

NuPAGE gel (Thermo Fisher, Halethorpe, MD) under reducing conditions. After transfer, the blot was incubated in blocking buffer (3% skim milk in PBS) for 1 h followed by incubation with rabbit anti-Oos-ANXL-2.1 serum (1:1000) in blocking buffer for 1 h at room temperature. The blot was washed five times with PBS containing 0.05% Tween-20 (PBST), incubated with goat anti-rabbit IgG-HRP (1:5000) for 1 h at room temperature, then washed again 5 times with PBST. The specific protein was visualized using a chemiluminescent substrate (SuperSignal® West Dura Exrended Duration Substrate, Thermo-Fisher, Rockford, IL, USA) according to the manufacturer's instructions. Beta-actin was monitored to control for loading. The same blot was stripped of antibodies in a buffer containing 1.5% glycine, 0.1% SDS, and 1% Tween 20, pH 2.2 (Abcam, www.abcam.com). The blot was washed twice in stripping buffer for 10 min at room temperature followed by 2 washes with PBS (pH 7.2) and PBST, respectively. After the final wash, the blot was treated with blocking buffer for 1 h at room temperature. The detection procedures with anti-beta actin antibody (mouse anti-bovine actin; Santa Cruz, CA) and secondary antibody (rabbit anti-mouse IgG-HRP; KPL, MD) were the same as described above. The image was captured and analyzed using a FluorChem E system (ProteinSimple, San Jose, CA, USA). The levels of integrated O.D. detected by the anti-Oos-ANXL-2.1 antibody for the protein bands were normalized in accordance with the levels of beta-actin. All data are expressed as beta actin-normalized integrated O.D. values: integrated O.D. = O.D. generated by anti-Oos-ANXL-2.1 antibody / O.D. generated by anti-beta actin antibody.

Immunolocalization of Oos-ANXL-2s in *O. ostertagi*

Immunolocalization of Oos-ANXL-2s protein in *O. ostertagi* was performed using the methods described elsewhere (Qu et al. 2014). Worms were harvested at different stages (i.e., L3, L4, and adult stages) of development, washed briefly in PBS, and fixed in 10% neutral formalin for 12 h. After fixation, *O. ostertagi* were embedded in paraffin and sectioned at 5 μm thickness for staining (HistoServe Inc., Germantown, MD). The sections were deparaffinized, quenched with 3% H_2O_2, and rehydrated. Antigen retrieval was achieved by pepsin digestion (0.4% pepsin in 0.01 N HCl) at 37 °C for 15 min. The slides were washed twice with 0.75% Brij 35 (Sigma) in PBS (BRIJ-PBS) and blocked with 0.5% sodium caseinate in BRIJ-PBS for 10 min. After blocking, the slides were incubated with the rabbit pre-immune or anti-Oos-ANXL-2.1 sera (1:1000) for 30 min at room temperature and then washed with PBS. After washing, goat anti-rabbit IgG-horseradish peroxidase (HRP) (1:500) and Dako AEC substrate chromogen (Dako, Carpinteria, CA, USA) were used to visualize bound antibody. The samples were counter-stained with Hematoxylin prior to applying a coverslip. Micrographs were taken using the Zeiss Axioskiope 2 Plus microscope (Zeiss, Thornwood, NY, USA).

Statistics

The integrated optical density data were analyzed by the GraphPad InStat software (La Jolla, CA, USA) using one-way ANOVA with Student-Newman-Keuls multiple comparisons test. Unless otherwise indicated, all data are presented as mean + SEM with $P < 0.05$ considered statistically significant.

Results

Identification of Oos-*anxl* transcripts

A total of 22 Oos-*anxl* genes were identified and retrieved from the database (http://nematode.net/NN3_frontpage.cgi) by BLASTN search using the bovine *anxa-1* (NP_786978, Table 1). Multiple sequence alignment of the 22 transcripts revealed that there are two distinct families of Oos-*anxl*, which were named Oos-*anxl-1* and Oos-*anxl-2*, respectively. At the amino acid sequence level, Oos-ANXL-1 is 41, 47, and 53% identical to bovine ANXA1, ANXA5, and ANXA7, respectively, and Oos-ANXL-2 is approximately 32–36, 38–43, and 29–33% identical to bovine ANXA1, ANXA5, and ANXA7, respectively (Fig. 1a). Oos-*anxl-1* had two transcripts (Table 1) with over 99% nucleotide sequence homology, encoding an identical Oos-ANXL-1 protein of 256 amino acids (aa). Oos-ANXL-1 has no predicted signal peptide and contains three predicted ANX domains and two potential N-glycosylation sites. Oos-ANXL-2 has 20 transcripts (Table 1) sharing sequence homology of greater than 98% of various lengths, some of which are incomplete at the N-termini.

The aa sequences of the bovine ANXA1, one copy of the Oos-ANXL1 and two copies of Oos-ANXL-2, were compared (Fig. 1a). Oost_isotig07314 and Oost_isotig07317, which belong to the Oos-ANXL-2 family, were 100% identical within the aligned regions; however, Oost isotig07314 was substantially longer at the N-terminus relative to Oost isotig07317 and contained a 22aa stretch that was not present in either Oos-ANXL-1 or in mammalian annexins (Fig. 1a, Table 1). Further, we were not able to identify this insertion in any other nematode species examined (data not shown). Oos-ANXL-1 and Oos-ANXL-2 proteins are only 38% identical.

Among the 20 Oos-*anxl-2* transcripts, only 8 of those have complete ORFs which encode 2 identical Oos-ANXL-2s and are distinguished by the 22 aa insertion (Fig. 1a; Table 1). Thus, we named the Oos-ANXL-2 without the insertion-Oost-ANXL-2.1, and the protein containing the insertion-Oos-ANXL-2.2 (Table 1). None of the Oos-ANXLs were predicted to encode a signal peptide. Theoretical pI and molecular masses are 8.89 and 19.5 kDa for Oos-ANXL-2.1 and 9.5 and

Table 1 Oos-ANXL transcripts identified by BLASTN search with the bovine ANXA1 (accession number NP_786978)

Annexin	Complete ORF		Incomplete ORF	
	Without insertion	With insertion	Without insertion	With insertion
Oos-ANXL-1	None	Oos-ANXL-1	None	None
	None	Oost_isotig10235 Oost_isotig10236	None	None
Oos-ANXL-2	Oos-ANXL-2.1	Oos-ANXL-2.2	TBD	TBD
	Oost_isotig07309 Oost_isotig07312 Oost_isotig07317 Oost_isotig07319	Oost_isotig07305 Oost_isotig07307 Oost_isotig07313 Oost_isotig07315	Oost_isotig07308 Oost_isotig07310 Oost_isotig07316 Oost_isotig07318 Oost_isotig07321 Oost_isotig07323	Oost_isotig07304 Oost_isotig07306 Oost_isotig07311 Oost_isotig07314 Oost_isotig07320 Oost_isotig07322

All Oos-*anxl* transcripts have a complete translated C-terminus. Oos-*anxl-1* only has 2 transcripts with no insertion when compared to the bovine ANXA1. There are 20 Oos-*anxl-2* transcripts, 8 of which have a complete ORF and 12 of which have an incomplete N-termini. Half of the Oos-ANXL-2s have a 22 aa insertion in comparison with the bovine ANXA1 (Fig. 1). The present study only characterized one Oost-ANXL-2, which is named Oos-ANXL2.1 that may represent the smallest of all Oos-ANXLs. The nomenclature for other Oos-ANXL2s will need to be updated when they are fully characterized

ANX annexin, *ORF* open reading frame, *ANXL* annexin-like, *TBD* to be determined

22 kDa for Oos-ANXL-2.2, respectively. Both Oos-ANXL-2.1 (aa 24–89 and aa 99–164) and Oos-ANXL-2.2 (aa 24–89 and aa 132–168) were predicted to have two annexin repeats, whereas other Oost-ANXL-2s with incomplete N-termini such as Oost_isotig07308 contain as many as 4–5 annexin repeats (data not shown). Oos-ANXL-2s have one predicted N-glycosylation site.

Oos-ANXL-1 is most similar to a *H. contortus* annexin repeat domain containing protein (HcoARDCP, CDJ94946) and the *C. remanei* Nex-2 (CreNEX-2) with 93 and 80% identities, respectively (Fig. 1b). However, Oos-ANXL-2s are most similar to HcoARDCP (CDJ82502) and the CreNEX-3 with 88 and 48% sequence identities, respectively (Fig. 1c).

Since the Oos-*anxl-2s* were more abundant than *Oos-anxl-1s*, we decided to clone and express the Oos-*anxl-2s* and characterize the Oos-ANXL-2s with a complete ORF.

Oos-ANXL-2 cloning, expression and purification, and antibody production

PCR primers designed to amplify Oos-*anxl-2.1* and *-2.2* which have a complete ORF. PCR amplification resulted in a product of 519 bp (Fig. 2a), which corresponds to the ORF encoding Oos-ANXL-2.1 with 172 aa. For reasons unknown, the ORF encoding the Oos-ANXL-2.2 was not amplified. The amplified cDNA sequence containing ORFs corresponding to Oost_isotig07312 or Oost_isotig07319 was used for cloning and expression. The PCR-amplified Oos-ANXL-2.1 ORF was cloned into pET28a (+) vector and expressed in *E. coli* BL21 (DE3). Recombinant Oos-AMXL-2.1 (rOos-AMXL-2.1) with an N-terminal HIS tag was only soluble in guanidinium HCL and was purified to homogeneity by Ni-NTA affinity chromatography (Qiagen). Purified rOos-AMXL-2.1 exhibited a molecular mass of approximately 21 kDa as analyzed by NuPAGE (Fig. 2b).

Western blot analysis of Oos-ANXL-2 at different developmental stages of *O. ostertagia*

Antisera to rOos-ANXL-2.1 should also recognize other members of Oos-ANXL-2, and thus, the Oos-ANXL detected by this antibody is collectively referred to as Oos-ANXL-2s unless the specific isoforms are further defined. The anti-Oos-ANXL-2.1 antibody detected Oos-ANXL-2s in *Ostertagia* lysates with three molecular masses, 52, 40, and 19 kDa (Fig. 3a). While we know that the 19 kDa band corresponds to Oos-ANXL-2.1 based on calculated molecular mass, the other two bands detected by the specific antibody are unknown. Since they are immunologically related and

Fig. 1 ANX amino acid (aa) sequence alignments. The aa sequence alignment between Oos-ANXLs (Oos-ANXL1: OOST_ISOTIG10235; Oos-ANXL2: OOST_ISOTIG07314; and OOST_ISOTIG07317) and bovine ANX A1 (NP_786978) (**a**), Oos-ANXL1 (OOST_ISOTIG10235), *C. remanei* Nex-2 (CreNEX2, XP_003110729) and *H. contortus* annexin repeat domain containing protein (ARDCP, CDJ94946) (**b**), and Oos-ANXL2 (Oos-ANXL2.2: OOST_ISOTIG07305; Oos-ANXL2.1: OOST_ISOTIG07317), CreNEX3 (XP_003106095) and HcoARDCP (CDJ82502) (**c**). All accession numbers are listed to left and all names of the products are listed to the right. # indicates that the aa sequence was truncated to show only the aligned portions of all sequences. Identical aa in sequence are indicated by an *asterisk* "*". For **a**, identical aa are annotated by "*" when there are two or more aa sequences in the alignment. Some Oost-ANXL sequences have an insertion of 22 aa (**a**, **c**, *underlined*) in comparison to the bovine ANX A1

A

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OOST_ISOTIG07314      ------------------------------------------------------------ Oos-ANXL2
OOST_ISOTIG07317      ------------------------------------------------------------ Oos-ANXL2
NP_786978             MAMVSEFLKQAWFIENEEQEYIKTVKGSKGGPGSAVSPYPTFNPSSDVEALHKAITVKGV BovANXA1
OOST_ISOTIG10235      ----------------------------MLGTPSVRPLPNFNANQDAEALRKAMKGLGC Oos-ANXL1
                                                  * * * **   * *** **    *

OOST_ISOTIG07314      --QKVIDILVKCNNAQRQMIRTPYKVRYGKDLENELRRELSGDLEDVILALMQTPTKRDV
OOST_ISOTIG07317      ------------------------------------------------------------
NP_786978             DEATIIEILTKRNNAQRQQIKAAYLQEKGKPLDEVLKKALLGHLEEVVLALLKTPAQFDA
OOST_ISOTIG10235      NNSKVISVLCGRTNWQRQEIAKAFKVMYGKDLIKDLKSELSGDFEDLILALMEPPAVYDA
                           *  *    * *** *        ** *   *   * *  *   ***   **  *

OOST_ISOTIG07314      LDLHRAMKGFGTDEKVLIEILASRSNEEIRAIRNTYYTTYDKSLEEAIWSDTSGDFRRML
OOST_ISOTIG07317      ------------------------------------------------------------ML
NP_786978             EELRAAMKGLGTDEDTLNEILASRTNREIREINRVYREELKRDLAKDIASDTSGDYEKAL
OOST_ISOTIG10235      KQLHKAMQGLGTKESVLIEIMTSRTNAQIAEICSVYQQLYRTTLERDLVGDTSGSFQRLL
                           ** * ** *  * **  ** *     *   *       *      ****     *

OOST_ISOTIG07314      VLLLQGSRDEYGIPQYHKAVKDAQQLLRASDKKSGQDKFDAYKILATASPSHLAYVYTEL
OOST_ISOTIG07317      VLLLQGSRDEYGIPQYHKAVKDAQQLLRASDKKSGQDKFDAYKILATASPSHLAYVYTEL
NP_786978             LSLAKGDRSEELAVNDDLADSDARALYEAGERRKGTDVNVFITILTTRSYPHLRRVFQKY
OOST_ISOTIG10235      VSLCAGGRDESNRTDQLRANQDARKLYSAGERRLGTDESCFNAILASNNFSQLRLIFDEY
                        *  * * *        *  **  *  *     * *       **       *

OOST_ISOTIG07314      ETISGYSIEKTIDREFSGDLKNLLLALVMVSQSKPKFFAHQIHNAIKTILRCKQRPKKIS
OOST_ISOTIG07317      ETISGYSIEKTIDREFSGDLKNLLLALVMVSQSKPKFFAHQIHNAIK------------
NP_786978             SKYSKHDMNKVLDLELKGDIEKCLTVIVKCATSQPMFFAEKLHQAMK------------
OOST_ISOTIG10235      EKVTKHSIEKAIEAEFSGDVKDGLLAVIAVARNRPAYFAKLLHEA---------------
                            *    *  **    *           *  **   * *

OOST_ISOTIG07314      TQINFKFPLGLGVRDRDLIRVLVSRAEIDLATIQMDFERLFNKSLEQVIRDECKGAYRDA
OOST_ISOTIG07317      ---------GLGVRDRDLIRVLVSRAEIDLATIQMDFERLFNKSLEQVIRDECKGAYRDA
NP_786978             ---------GIGTRHKTLIRIMVSRSEIDMNDIKACYQKLYGISLCQAILDETKGDYEKI
OOST_ISOTIG10235      ------------------------------------------------------------
                            * * *   ***  *** ***   *      *   ** * * ** ** * *

OOST_ISOTIG07314      LLSIVKGNRSLY
OOST_ISOTIG07317      LLSIVKGNRSLY
NP_786978             LVALCGRD----
OOST_ISOTIG10235      ------------
                      *       ****
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B

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XP_003110729          #NHGYGQPVMIGTPSLFPIPGFNANADAEVLRKAMKGLGCNNSKVISVLCQRTNGQRQEI CreNEX2
OOST_ISOTIG10235       --------MLGTPSVRPLPNFNANQDAEALRKAMKGLGCNNSKVISVLCGRTNWQRQEI Oos-ANXL1
CDJ94946              #----MASEMLGTPSVRPVPNFNANQDAETLRKAMKGLGCNNSKVISVLCGRTNWQRQEI HcoARDCP
                             *:****: *:* **** ***.******************** *** *****

XP_003110729          SKAFKVMYGKDLIKELKGELHGDFEDLILALMEAPAIYDAKQLYKAMDGLGTKESVLIEI
OOST_ISOTIG10235      AKAFKVMYGKDLIKDLKSELSGDFEDLILALMEPPAVYDAKQLHKAMQGLGTKESVLIEI
CDJ94946              AKAFKVMYGKDLIRDLKSELSGDFEDLILALMEPTAVYDAKQLHKAMQGLGTKESVLIEI
                      :************::**.** ************  *:******:***:************

XP_003110729          MTSRTNAQIQQVRDAYKMLYKKDLERDLIGETSGHFKRLLVSLCAGGRDESNQTDALRAN
OOST_ISOTIG10235      MTSRTNAQIAEICSVYQQLYRTTLERDLVGDTSGSFQRLLVSLCAGGRDESNRTDQLRAN
CDJ94946              LTSRTNSQIAEIRNVYQQLFRTSLENDIIGDTSGCFQRLLVSLCAGGRDESNRTDPLRAN
                      :*****:** :: ..*: *::. **.*::*:*** *:***************:** ****

XP_003110729          QDARRLYQAGEKRLGTDESTFNAILASQNFNQLRMVFEEYQKVSNHSIEKAIESEFSGDV
OOST_ISOTIG10235      QDARKLYSAGERRLGTDESCFNAILASNNFSQLRLIFDEYEKVTKHSIEKAIEAEFSGDV
CDJ94946              QDARKLYSAGERRLGTDESCFNAILASNNFSQLRLIFDEYQKVTNHSIEKAIEAEFSGDV
                      ****:**.***:******* *******:**.***::*:**:**::********:******

XP_003110729          RDGLLAVIAVVRNRPAYFAKLLHDSMKGLGTRDNDLIRLCVTRAEYDMADIRNMFQSLYR
OOST_ISOTIG10235      KDGLLAVIAVARNRPAYFAKLLHEA----------------------------------
CDJ94946              KDGLLAVIAVARNRPAYFAKLLYESMKGFGTRDSDLIRLVVTRCEYDMVDIRSAFQAMYK
                      :*********.**********:::

XP_003110729          TTLENMIKGDCSGAYKEGLIALVNGNRGTM
OOST_ISOTIG10235      -----------------------------
CDJ94946              TTLENMIKGDCSGSYKDGLIALVTGNAHAH
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C

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XP_003106095,         #TKNTYFMTYGKSLEDAISADTSGDFRRLLIIILQAKREETGFANTFNVGNHAAQILKSF CreNEX3
CDJ82502              #IRNTFYTTYDKSLEETIWSETSGDFRRMLVLLIQGTRDEYGIPQYHKAVQDAQQIMRAC HcoARDCP
OOST_ISOTIG07305      ----------------------------MLVLLLQGSRDEYGIPQYHKAVKDAQQLLRAS Oos-ANXL2.2
OOST_ISOTIG07317      ----------------------------MLVLLLQGSRDEYGIPQYHKAVKDAQQLLRAS Oos-ANXL2.1
                                                  :*::::*..*:* *: : .:. :.* *::::

XP_003106095,         DKKSGIEKFDAFKIFATSNGAHIQKVIEEVERQSGKDFQKIADKELSGDFKNLILSLIET
CDJ82502              DKKSGQDKFEAFKILATASPSHLAYVYSELETISGYSIEKTIDHEYSGDMKTLLLALVMV
OOST_ISOTIG07305      DKKSGQDKFDAYKILATASPSHLAYVYTELETISGYSIEKTIDREFSGDLKNLLLALVMV
OOST_ISOTIG07317      DKKSGQDKFDAYKILATASPSHLAYVYTELETISGYSIEKTIDREFSGDLKNLLLALVMV
                      ***** :**:*:**:**:. :*:  *  *:*  ** .::*  *:* ***:*.*:*:*: .

XP_003106095,         SSNKPRFLANSIHLATK----------------------GIGTRDKDLIRILVARSENDL
CDJ82502              SQSKPKFFAHQIHNAIK----------------------GLGVRDKDLIRVLVSRAETDL
OOST_ISOTIG07305      SQSKPKFFAHQIHNAIKTILRCKQRPKKISTQINFKFPLGLGVRDRDLIRVLVSRAEIDL
OOST_ISOTIG07317      SQSKPKFFAHQIHNAIK----------------------GLGVRDRDLIRVLVSRAEIDL
                      *..**:*:*..** * *                     *:*.**:****:**:*:* **

XP_003106095,         VLIEHEFQVLFGKPLTQLIKDECKAEYRDGLTMLVKGNSRP-
CDJ82502              ATIQMEYERLFNKSLEQVIRDECKGDYRDALLRIVKGNRSRY
OOST_ISOTIG07305      ATIQMDFERLFNKSLEQVIRDECKGAYRDALLSIVKGNRSLY
OOST_ISOTIG07317      ATIQMDFERLFNKSLEQVIRDECKGAYRDALLSIVKGNRSLY
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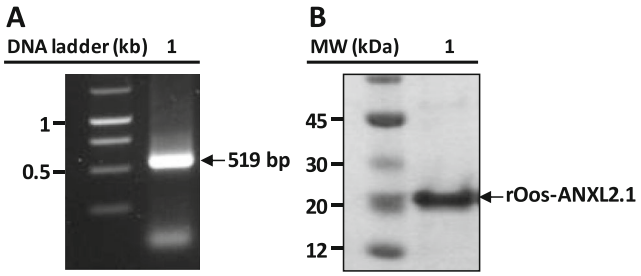

Fig. 2 PCR-amplification of Oos-ANXL2.1 (**a**, *lane 1*) and recombinant Oost-ANXL2.1 (rOos-ANXL2.1) produced in *E. coli* (**b**, *lane 1*). **a** PCR product was analyzed by a 1% agarose gel, stained by ethedium bromide, and photographed. **b** Purified rOos-ANXL2.1 was separated on a 4–12% gradient SDS-PAGE (NuPGAE, Thermo Fisher), stained with Coomassie blue, and photographed

potentially encoded by Oos-ANXL-2 transcripts with undefined N-termini, the two unknown bands were designated band-1 (Oos-ANXL-2-b1, 52 kDa) and band-2 (Oos-ANXL-2-b2, 40 kDa) (Fig. 3a). The rOos-ANXL-2.1 with an N-terminal HIS-tag had a molecular mass of 21 kDa. For

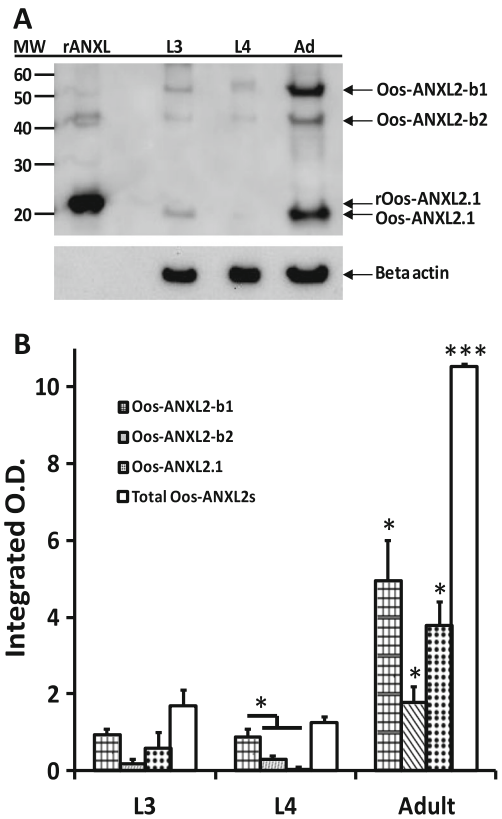

Fig. 3 Western blot analysis of Oos-ANXL2s in *O. ostertagi* at different developmental stages. **a** Representative Western blot analysis of Oos-ANXL2 in *Ostertagia* L3, L4, and adult worm. **b** Semi-quantitation of Oos-ANXL2s in *Ostertagia* larval and adult worm lysates. *Ostertagia* beta-actin was detected by an anti-bovine beta-actin antibody (Santa Cruz). Data represent results obtained from three independent experiments. Oos-ANXL2-b1 and b2, band-1 and band-2 of Oos-ANXL2; Oos-ANXL2.1, native Oos-ANXL2.1. *L3* stage 3 larvae, *L4* stage 4 larvae, *Ad* adult stage of the parasite, *MW* molecular mass, *rANXL* recombinant Oos-ANXL2.1. *$P < 0.05$; **$P < 0.01$

comparison purposes across different samples, the integrated O.D. values for Oost-ANXL-2s of each of the test samples were normalized by the integrated O.D. values for beta-actin. Across different developmental stages, levels of individual Oos-ANXL-2s ($P < 0.05$) or total Oos-ANXL-2 (sum of the 3 Oost-ANXL-2 bands) ($P < 0.001$) were significantly higher in adult worm lysates than in L3 or L4 lysates (Fig. 3b). Within each developmental stage, levels of the 3 Oos-ANXL-2 isoforms did not differ ($P > 0.05$) for L3 and adult stages; however, in the L4, levels of Oos-ANXL-2-b1 were significantly higher ($P < 0.05$) than those of Oos-ANXL-2-b2 or Oos-ANXL-2.1 (Fig. 3b).

Immunolocalization of Oos-ANXL-2s in *O. ostertagi*

Oos-ANXL-2s were clearly detected in the L3 and adult stages (Fig. 4b, c, f, g), but appeared more abundant in the adult stage. Oos-ANXL-2s were very low or undetectable in L4 larvae (Fig. 4d, e). The staining in L3 seemed to be diffuse, and it was difficult to pin-point if the Oos-ANXL-2s were present in the hypodermis, gut, or the reproductive tract (Fig. 4b, c). However, staining in the adult was clearly and specifically localized to the hyperdermis, but not in cuticle, muscle, gut, or the reproductive tract (Fig. 4f, g).

Discussion

Mammalian annexins are a family of proteins characterized by membrane binding and shared functions in membrane-related processes (Gerke et al. 2005). Structurally, these ANXs typically contain 4 core repeats of 70 amino acids (van Genderen et al. 2008). Among the family members, ANXA1 has been characterized as an anti-inflammatory protein capable of down-regulating inflammation reactions. Human annexin A1 is regulated by glucocorticoid (GC) (Ahluwalia 1998; Buckingham et al. 2003). In general, ANX-A1 functions in vivo as an endogenous suppressor of inflammation in cells of the innate immune system. Once externalized on the cell surface of adherent/activated neutrophils, it blocks the interaction with endothelial cells. Animals treated with exogenous recombinant ANX-A1 have reduced neutrophil adhesion and emigration, and local leukocyte recruitment is inhibited (van Genderen et al. 2008; Gavins and Hickey 2012).

Twenty-two Oos-*anxl* transcripts (http://nematode.net/NN3_frontpage.cgi) have been aligned with the bovine ANXA1 in the present study. Oos-*anxl*-1 and -2 transcripts are likely derived from two separate gene families. There may be two explanations for multiple transcripts within each of the Oos-*anxl* families; one is the presence of multiple genes or differences between parasite strains. Indeed, all parasite sequences in the database were derived from mixed populations of worms (Heizer et al. 2013). This is further supported by our effort to clone Oos-ANXLs where of

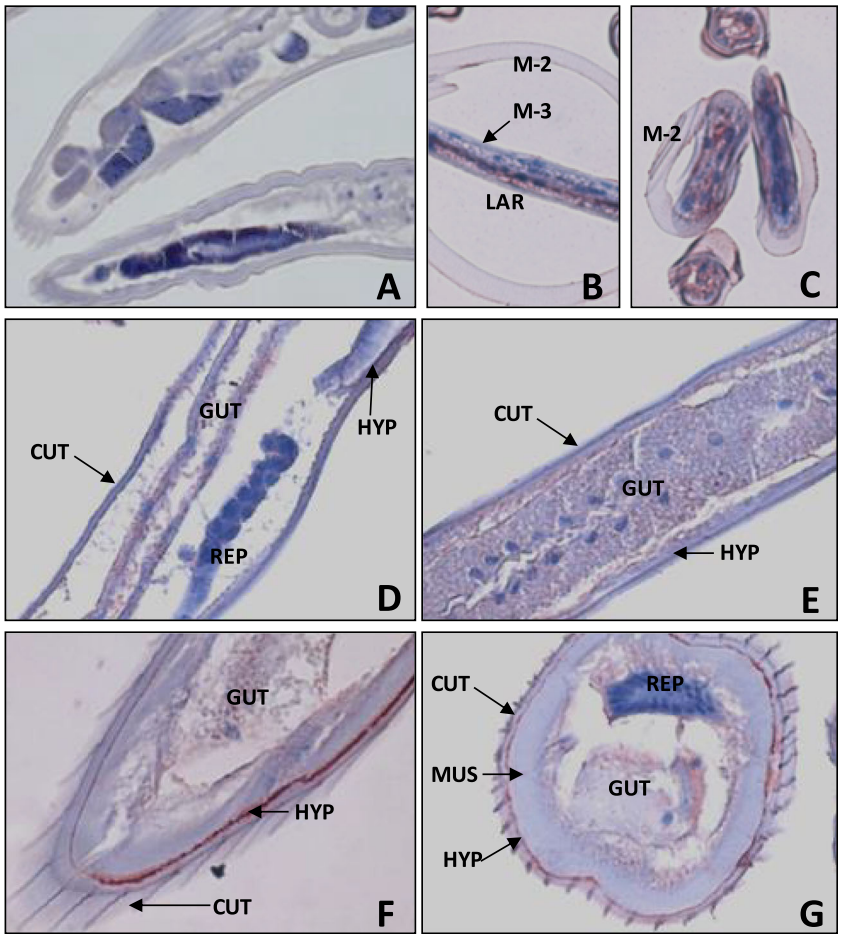

Fig. 4 Immunolocalization of Oos-ANXL2s in *O. ostertagi*. **a** Adult *O. ostertagi* stained with pre-immune serum as a negative control. **b**, **c** L3 stained with anti-Oos-ANXL2.1 antibody. **d**, **e** L4 stained with Oos-ANXL2.1 antibody. **f**, **g** Adult *O. ostertagi* stained with Oos-ANXL2.1 antibody. *M-2* second molt cuticle, *M-3* third molt cuticle, *LAR* larva, *CUT* cuticle, *GUT* gut, *REP* reproductive tract, *HYP* hyperdermis, *MUS* muscle. Magnification: 400×

the eight clones sequenced, six possessed mutations, all of which were synonymous. BLASTN search of the NCBI bovine protein database with either Oos-ANXL-1 or -2 amino acid sequences showed that Oos-ANXL-1 shares slightly higher sequence identity with the bovine ANXA7 (53%) than does Oos-ANXL-2 with bovine ANXA5 (43%); however, both families nonetheless share less identity (36–41%) with bovine ANXA1. In addition, Oos-ANXL-1 and Oos-ANXL-2 are homologs of CreNEX2 and CreNEX3, respectively. The present study has characterized an Oos-ANXL protein that represents the most abundant family of the Oos-ANXLs, the Oos-ANXL-2 (Table 1). It is unknown why we were not able to PCR-amplify the transcript encoding Oos-*anxl-2.2* (with 22 aa insertion) using the same pair of primers used to amplify Oos-*anxl-2.1*.

Even though we were not able to PCR-amplify Oos-*anxl*-2.2, the anti-Oos-ANXL-2.1 antibody produced in rabbit should recognize other Oos-ANXL-2 family members due to high sequence homology. The Western blot data suggests that immunoreactive Oos-ANXL-2s exist in three molecular sizes in parasite lysates and are differentially produced among the developmental stages. Based on the molecular mass of Oos-ANXL-2.1, this is likely represented by the protein migrating at an apparent molecular mass of 19 kDa in Western blots. However, the other two bands (b1 and b2) with molecular masses of 40 and 52 kDa could represent products of the Oos-*anxl-2* transcripts with longer N-termini. Surprisingly, the predicted Oos-ANXL-2.2 protein (with 22 aa insertion) which should have a molecular mass slightly higher than that of Oos-ANXL-2.1 was not detected by the anti-Oos-ANXL-2.1 antibody in Western blot. Taken together, this suggests that Oos-ANXL-2.2 may not exist based on the lack of PCR-amplifiable transcript and the protein product. Again, this will need to be further confirmed when the *Ostertagia* genome is available. Overall, production of either Oos-ANXL-2 bands (b1, b2, or Oos-ANXL-2.1) or total Oos-ANXL-2s was the highest in adult *Ostertagia* and lowest in the L4; this was confirmed by immunohistochemistry.

Oos-ANXL-2s were localized to L3 *Ostertagia* larvae as well as adult *Ostertagia*. The proteins were clearly localized to the hypodermis, but not to muscle, gut, or reproductive tract in the adult worm. Previous studies showed that *C. elegans* annexins (NEX-1 to 4) had different temporal and spatial distributions (Nishioka et al. 2007). It was found that *nex-1* and *-3* were transcribed continuously during all developmental stages whereas NEX-2 and -4 were developmentally regulated, with the most abundant transcripts appearing in the L1

stage (Nishioka et al. 2007). Interestingly, the same study also showed that the *C. elegans* NEX-2 existed in multiple molecular masses. Obviously, the temporal and spatial distribution of Oos-ANXL-2s is different from that *C. elegans* annexins, suggesting that Ostertagia annexins may function differently from those in the free-living nematodes.

During ostertagiosis, the parasite stays immobile in the gastric glands until it reaches late L4 or young adult when it becomes mobile and actively exits the gastric glands (Fox 1997). It is believed that the exiting process physically causes damages to the abomasum, which can be very severe particularly in type II ostertagiosis. The inflammation caused by the tissue damage and associated host inflammation can be hostile to the parasite's survival, and therefore, it is possible that the parasite produces immune modulators during the adult stage to down-regulate host responses that are detrimental to parasite longevity.

Potential roles for Oos-ANXL-2s were not investigated in the present study because the recombinant Oos-ANXL-2.1 was produced in bacterial inclusion bodies and only soluble in 8 M urea or guanidinium HCL, suggesting that the recombinant protein was likely mis-folded. Future studies will focus on production of recombinant Oos-ANXLs in a different expression system or purification of native Oos-ANXLs from the worm lysates using conventional and/or affinity-based chromatography.

Based on what has been documented in the databases, Oos-ANXLs are not as complex as those in mammals. In fact, *O. ostertagi* may only have two major families of annexins which are fewer that those in the free-living nematodes *C. elegans* or *C. remanei*. As shown in sequence alignment, Oos-ANXLs are moderately conserved with 29–53% similarities with more than 12 members of the mammalian annexins. Therefore, it is very difficult to assign the Oos-ANXLs to any of the characterized mammalian annexins without demonstrating the biological functions of these proteins. However, it may be speculated that, as a result of evolution, certain specialized roles of annexins have been assigned to and carried out by multiple specialized annexin isoforms in mammals. In nematode such as *O. ostertagi*, the multiple annexin-specific functions of this organism may be carried out by only two annexin family members. The Oos-ANXLs as potential immunosuppressive immune modulator proteins could be vaccine targets against *O. ostertagi* infection and warrant further investigation.

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